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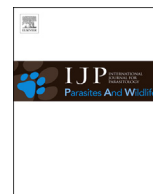


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First description of *Onchocerca jakutensis* (Nematoda: Filarioidea) in red deer (*Cervus elaphus*) in Switzerland



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ABSTRACT

Twenty-seven species of the genus *Onchocerca* (Nematoda; Filarioidea) can cause a vector-borne parasitic disease called onchocercosis. Most *Onchocerca* species infect wild and domestic ungulates or the dog, and one species causes river blindness in humans mainly in tropical Africa. The European red deer (*Cervus e. elaphus*) is host to four species, which are transmitted by blackflies (simuliids) or biting midges (ceratopogonids). Two species, *Onchocerca flexuosa* and *Onchocerca jakutensis*, produce subcutaneous nodules, whereas *Onchocerca skrjabini* and *Onchocerca garmsi* live free in the hypodermal serous membranes. During the hunting season, September 2013, red deer ($n = 25$), roe deer (*Capreolus c. capreolus*, $n = 6$) and chamois (*Rupicapra r. rupicapra*, $n = 7$), all shot in the Grisons Region (Switzerland) were investigated for the presence of subcutaneous nodules which were enzymatically digested, and the contained *Onchocerca* worms were identified to species by light and scanning electron microscopy as well as by PCR/sequencing. In addition, microfilariae from skin samples were collected and genetically characterized. Neither nodules nor microfilariae were discovered in the roe deer and chamois. Adult worms were found in 24% of red deer, and all of them were identified as *O. jakutensis*. Two morphologically different microfilariae were obtained from five red deer, and genetic analysis of a skin sample of one red deer indicated the presence of another *Onchocerca* species. This is the first report of *O. jakutensis* in Switzerland with a prevalence in red deer similar to that in neighbouring Germany.

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1. Introduction

Onchocercosis is a parasitic disease caused by any of the 27 species of the genus *Onchocerca* which are transmitted by simuliid or ceratopogonid insect vectors (Anderson, 2000). Most *Onchocerca* spp. infect wild and domestic ungulates, while *Onchocerca lupi* Rodonaja, 1967 infects dogs. The parasites cause lesions, inflammation of musculature and joints and lameness in ungulates, or eye disease in dogs (Deplazes et al., 2013). Furthermore, *Onchocerca volvulus* (Leuckart, 1893) Railliet and Henry, 1910 is the causative agent of river blindness of humans, mainly in tropical Africa, with currently over 37 million people being infected (WHO, 2015). Except for river blindness, onchocercosis in humans is rare. Only 25 cases have been described to date (reviewed in Uni et al., 2015),

such as an unusual infection with *Onchocerca jakutensis* in a woman in Austria (Koehsler et al., 2007).

Worldwide seven *Onchocerca* species are known to parasitize Cervidae (Anderson, 2000). Publications from Europe describe four species in red deer (*Cervus elaphus*) in central Europe: *Onchocerca flexuosa* (Wedl, 1856), *O. jakutensis* (Gubanov, 1964) (Syn. *Onchocerca tubingensis* Bain and Schulz-Key, 1974), *Onchocerca skrjabini* (Ruchljadev, 1964) (Syn. *Onchocerca tarsicola* Bain and Schulz-Key, 1974) and *Onchocerca garmsi* Bain and Schulz-Key, 1976 (Schulz-Key et al., 1975). Studies have revealed *Onchocerca* infections in cervids in the Czech Republic and Slovakia (Dyková and Blazek, 1972), Hungary (Meszaros and Sugar, 1974), Yugoslavia (Brglez and Zeleznik, 1976), Romania (Dulceanu and Ghitescu, 1986), Germany (Schulz-Key, 1975), Poland (Demaskiewicz, 1993), Spain (Santín-Durán et al., 2001; San-Miguel et al., 2003) and Italy (Morandi et al., 2011).

Two species, *O. flexuosa* and *O. jakutensis*, produce large subcutaneous nodules up to several centimetres in diameter, whereas *O. skrjabini* and *O. garmsi* live free in the hypodermal serous membranes (Plenge-Bönig et al., 1994). The subcutaneous nodules

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of *O. flexuosa* are located between the shoulder blade and the lumbar region (Brglez et al., 1967), in the region of the sacrum and rarely on the lateral abdomen or proximal parts of the extremities (Plenge-Bönig et al., 1994). The subcutaneous nodules of *O. jakutensis* are found in the region of the external thigh or in the caudal part of the back (Bain and Schulz-Key, 1974). The rare filarial species *O. garmsi* is located in the tissue above the sternum (Schulz-Key et al., 1975). *Onchocerca skrjabini* is found free between the serous membranes above the radio-carpal and tibio-tarsal joints or on the abductor tendon (Bain and Schulz-Key, 1974).

The microfilariae of *O. garmsi* and *O. jakutensis* are present with highest concentrations in the skin above the sternum, and also at the base of the ear. Microfilariae of *O. skrjabini* are predominantly found in the exterior part of the ear. They also appear in the nose region, on the scrotum and on the insides of the thigh (Schulz-Key et al., 1975), whereas preferred sites of *O. flexuosa* microfilariae are the inside of the thigh and the distal parts of the hind leg (Schulz-Key, 1975).

Little is known about the pathogenicity of the different species of *Onchocerca* in red deer. Dyková (1970) described a chronic sclerosing lymphadenitis caused by microfilariae of *O. flexuosa*. Later, she observed a microfilaria-induced myositis of the surrounding musculature and a dystrophic change of the hypodermal tissue (Dyková, 1972). Commichau (1982) reported a polyarthritis caused by an *Onchocerca* species in red deer.

The present investigation elucidates the presence of *Onchocerca* spp. in wild ruminants in Switzerland. The presence as well as the particular species is also of medical interest, as endemic zoonotic onchocercosis should be considered. Furthermore, the morphology of *O. jakutensis* as described by Bain and Schulz-Key (1974) versus Demaskiewicz (1993) differs in some parts. In such situations, DNA analysis has proven helpful and was used e.g. by Morandi et al. (2011) and in a zoonotic case (Koebsler et al., 2007). However, the morphology of the specimens identified in these molecular analyses remains uncertain. A detailed morphological description of the worms recovered here is therefore included in the present investigation to link with the results of DNA analyses.

2. Materials and methods

2.1. Origin of samples, sample preparation and morphological identification

The skins of 25 free-ranging red deer (*Cervus e. elaphus*), six roe deer (*Capreolus c. capreolus*) and seven chamois (*Rupicapra r. rupicapra*) were investigated for nodules of adult worms during the hunting season, September 2013. The animals were shot in the Grisons Region, Switzerland (Churer Rheintal, Prättigau, Heinzenberg, Albulatal, Hinterrheintal, Lenzerheide, Safiental, Domleschg and Misox). To isolate adult worms, the nodules were incubated for 8 h at 60 °C in subtilisin-enzyme solution (10% [v/v] Enzyrim OSA, Bauer Handels GmbH, Adetswil, Switzerland), buffered at pH 8.00 (9888 Titrisol Merck, Germany) and 2 drops of Mollescal-C (Bauer Handels GmbH) per 10 ml. After lysis specimens were fixed and stored in 70% ethanol. For morphological examination, adult worms were cleared in chloro-lactophenol. Drawings were done with a Leitz drawing tube. Scanning electron microscopy was performed at the Center for Microscopy and Image Analysis, University of Zurich using standard procedures. Specimens were dehydrated in 100% ethanol, followed by critical-point-drying and then coated with carbon or platinum.

In addition, 2 × 2 cm skin samples from the abdominal and from the metatarsal regions were collected from all investigated ungulates. The skin samples were treated as described by Schulz-Key (1975), with some modifications: They were cut into small pieces

and incubated in petri dishes for 2–5 h at 37 °C in 20 ml of a saline solution containing 0.9% (w/v) NaCl and 0.1% (v/v) Mollescal-C (BASF, Ludwigshafen, Germany) as a preservative. After the incubation period, the skin shreds were removed, the petri dish set at a slight angle, and 8 droplets of the sediment microscopically investigated for the presence of microfilariae. The rest of the sediment from the samples containing microfilariae were kept frozen in 1.5 ml Eppendorf tubes for genetic analyses. Morphological identification was achieved using the keys given in Bain (1981) and Demaskiewicz (1993).

2.2. DNA extraction

About 2 cm long sections of single adult worms (n = 10 from four red deer) were washed 2 to 3 times in phosphate-buffered saline (PBS) at room temperature and transferred into 1.5 ml Eppendorf tubes containing 20–40 µl PBS. To disrupt the cell membranes, the tubes were frozen in liquid nitrogen, thawed in a heat block at 100 °C for 1 min and then rigorously vortexed for 1 min. This step was repeated three times. Worms were then triturated with a tissue homogenizer as described by Wenk et al. (2012). The tubes containing microfilariae were centrifuged for 5 min and the supernatant was reduced to 30 µl. Five µl were again examined for the presence of microfilariae, and the remaining 25 µl were minced in a tissue homogenizer. DNA isolation was done with the Qiagen (Hildesheim, Germany) Qiaamp DNA mini kit according to the tissue protocol. DNA isolations were immediately frozen on dry ice and stored at –18 °C.

2.3. PCR

PCR was carried out according to Morales-Hojas et al. (2006), targeting the mitochondrial NADH-dehydrogenase gene. Amplifications were performed using the forward primer ND50vA (5'-TTG GTT GCC TAA GGC TAT GG-3') and the reverse primer ND50vC (5'-CCC CTA GTA AAC AAC AAA CCA CA-3') in a MyCycler thermocycler (Biorad, Cressier, Switzerland). Reactions were performed in a total volume of 20 µl, containing standard Taq-polymerase buffer (BioConcept, Alschwil, Switzerland), 200 µM each dNTP (NBL, UK), 0.4 µM each primer (Microsynth, Balgach, Switzerland), 1 unit Taq-DNA polymerase (BioConcept) and 2 µl of the DNA extract. Amplifications consisted of an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of 30 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C, with a final extension step of 5 min at 72 °C. Amplicons were analysed on 3% agarose gels, stained with GelRed™ (Chemie Brunschwig, Basel, Switzerland) and visualized with UV light. Negative controls containing H₂O were run to control for contamination.

2.4. Sequencing and analysis

Amplicons were sequenced (Synergene, Schlieren, Switzerland), after purification with the minelute PCR purification kit (Qiagen) following the manufacturer's instructions, using the PCR forward primer and 5 µl of the amplicons. Sequences were aligned with Multialin (Corpet, 1988) and then compared with GenBank entries.

3. Results

3.1. Prevalence of *O. jakutensis*

Of the 38 artiodactyls from the three species examined, *Onchocerca* specimens were found only in red deer. In six of the 25 red deer, subcutaneous nodules (n = 13) (Fig. 1) were collected containing female and male or only female *O. jakutensis* worms as

identified by morphological and genetical analyses (prevalence 24%). Up to five nodules were found per deer. Nearly half of the collected adult *Onchocerca* worms contained granular lumps (Fig. 2) or were in a state of advanced decay to calcification. Microfilariae were observed in eight of ten tissue samples from five red deer from which adult *O. jakutensis* were recovered from only three.

3.2. Morphology of *O. jakutensis*

The living worms are transparent opalescent, white-yellowish filarioids. Females are much longer than males. Mouth orifice 1.5 μ m in diameter. Oesophagus divided in anterior muscular part and slightly longer posterior glandular part.

3.2.1. Male of *O. jakutensis* (Fig. 3a–d)

The description is given for the same specimen which was also used for PCR/DNA sequencing and SEM. (In addition measurements of another entire male and of the posterior part of a further male specimen are given in parentheses).

Body length 36 mm (40 mm), tapering gradually within about 6 mm towards anterior end, from a width of 180 μ m (160 μ m, 170 μ m) at midbody to 90 μ m (90 μ m) at end of oesophagus and more rapidly tapering to 60 μ m (50 μ m, 50 μ m) in diameter at cloaca. Cuticle with thin transverse annulations, 2.5 μ m apart at midbody, narrowing towards both ends. Oesophagus length 670 μ m (530 μ m), glandular posterior part 360 μ m (280 μ m). Nerve ring close to end of muscular oesophagus at 250 μ m (220 μ m) from anterior end (Fig. 3d). Tail 130 μ m (110 μ m, 110 μ m) long, coiled ventrad, flattened or slightly concave on ventral side (Fig. 3a).

Spicules unequal and dissimilar. Left spicule 275 μ m (260 μ m, 220 μ m), right 90 μ m (90 μ m, 110 μ m) long, both with well developed head. Proximal shaft cylindrical, distal part modified in both. Left spicule: distal part slightly longer than shaft, thinned to membrane with margins bent ventrad to form tube, very fine membrane forming pointed tip of spicule. Right spicule: distal half flattening to concave groove with strong knob on dorsal distal end. In protruded position, the right spicule serves as gubernaculum for the left spiculum. No gubernaculum present (Fig. 3b–c).

Two phasmids ventro-lateral near tip of tail with conspicuously protruding flaps. Five pairs of pericloacal papillae and 2 pairs close to tail tip, in proximity to phasmids (Fig. 3a–b). No unpaired



Fig. 2. Anterior end of *O. jakutensis* female, with granular lumps in process of degeneration.

precloacal papilla present (Fig. 4a,b). Considerable individual variation in placement and size of papillae (note in Fig. 3b the two very small papillae in pericloacal group and the distal pair on tail tip). In two of three specimens one pair of papillae set between cloacal group and group on tail tip.

3.2.2. Female of *O. jakutensis* (Fig. 3e–f)

Description is given of 4 entire females. In addition measurement of the specimen which was also used for PCR/sequencing is given in parentheses.

Body length 705–780 mm (810 mm). Oesophagus 600–800 μ m (720 μ m), glandular part slightly longer than anterior, muscular part. Vulva posterior to oesophagus, 1260–1500 μ m (870 μ m) from anterior end (Fig. 3e). Body diameter 310–320 μ m (310 μ m) at midbody, gradually tapering in anterior part to 120–130 μ m (130 μ m) diameter at end of oesophagus, tapering more rapidly posteriad to 65–105 μ m (110 μ m) diameter at anus. Tail nearly cylindrical, 90–165 μ m (170 μ m) long with rounded tip, bearing two flapped phasmids subapically (Fig. 3f).

Outermost layer of cuticle thickened in intervals to conspicuous transverse annulations, which fade out towards lateral fields. Annulations rough in appearance, forming jagged lines (Fig. 5). Some single annuli bifurcated or interrupted towards lateral fields. Internal medullar part of cuticle forming transversely oriented, cushion-like striae, with the appearance of square cells in transverse sections. External annuli to striae in the ratio 1:4 (Fig. 6). External annulation smoothing out towards head and tail end of body, becoming smaller and narrower as in males (Fig. 3f).

3.2.3. Description of microfilariae from *O. jakutensis* from uterus

Microfilaria without sheath. Long and thin, specimens measured in oviduct close to vulva 260–270 μ m long, 6 μ m wide. Cuticle with fine transverse annulation, 0.5 μ m apart (Fig. 7). Anterior end unevenly rounded with subapical protrusion, in some microfilariae slightly swollen (Fig. 3g). Tail tapering to fine tip. The distribution of terminal nuclei is depicted in Fig. 3g.

3.3. Sequences

PCR from adult worms ($n = 10$) yielded amplicons of around 440 bp. They were identical except for one sequence that differed at one nucleotide position from all other sequences (GenBank accession nrs. KU886066, KU886067). The sequences had a very high identity (399 of 401 nucleotides) with *O. jakutensis* sequences from

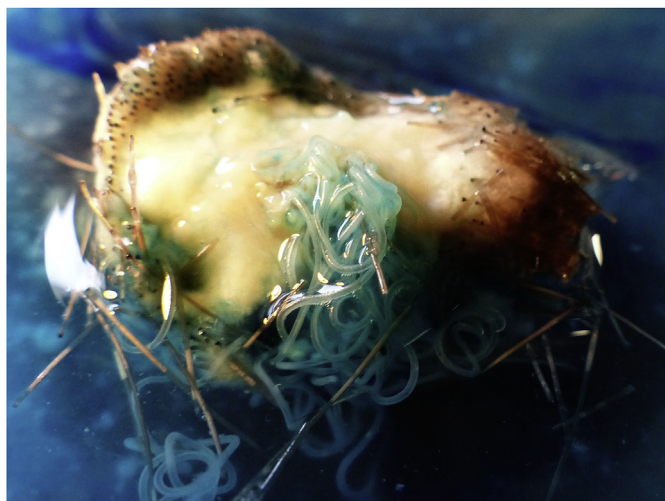


Fig. 1. Subcutaneous nodule in red deer skin with partly freed *O. jakutensis* female (stained with methylene blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

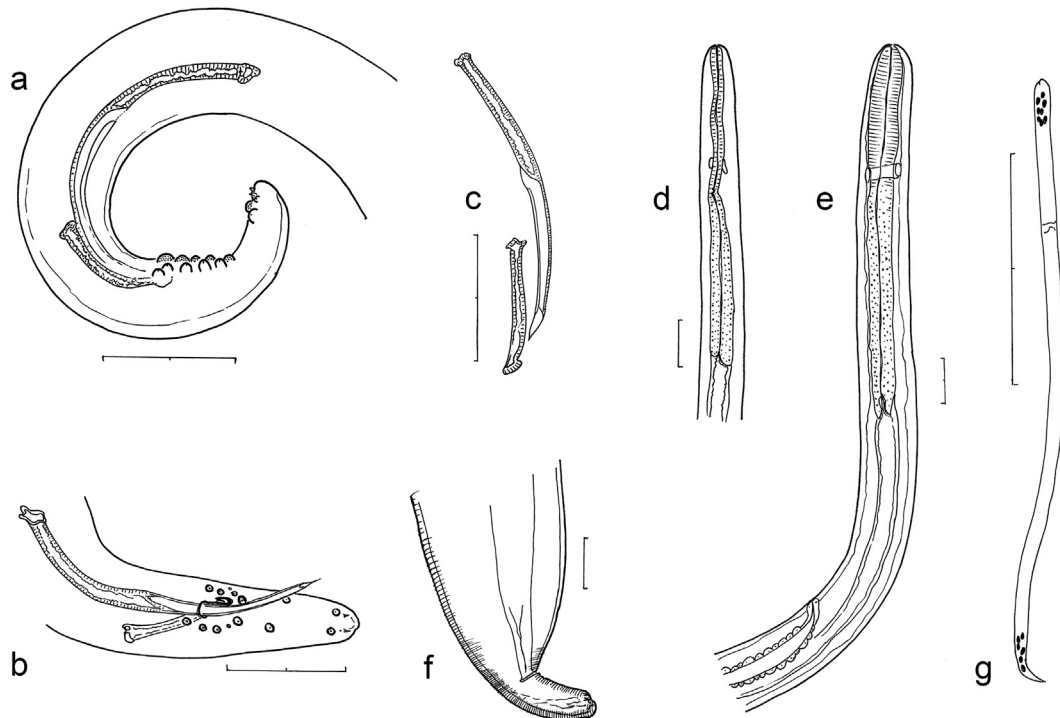


Fig. 3. *O. jakutensis* male and female (bars: 100 µm): a. Tail of male with five pairs of pericloacal and two pair of closely spaced terminal papillae. b. Tail of another male with more distantly spaced papillae on tail end. Left spicule protruding. c. Spicules in ventral view. d. Head end of male. e. Head end of female with vulva. f. Posterior end of female with conical tail in ventral view, annulations indicated on sides. g. Microfilaria with terminal distribution of nuclei.

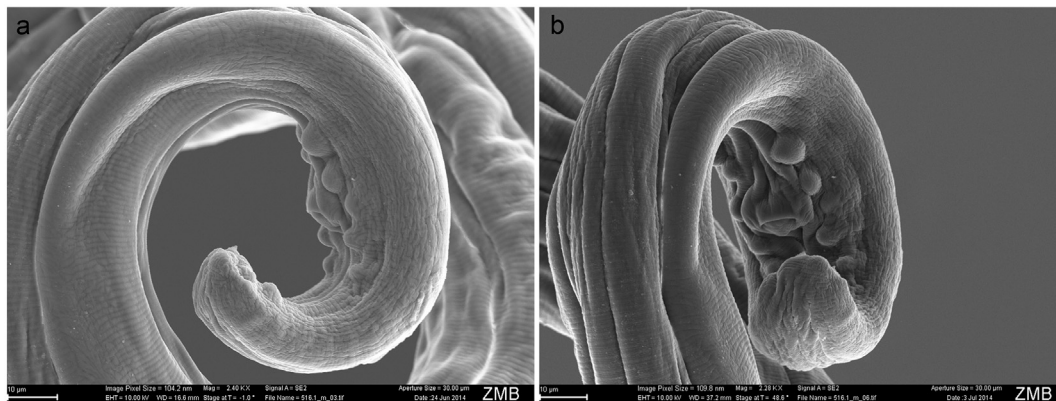


Fig. 4. a and b: Posterior end of *O. jakutensis* male with 5 pairs of pericloacal papillae without unpaired preloacal papilla.

Germany (Genbank accession: DQ523771) with <1% difference (two substitutions).

PCRs with DNA isolated from six microfilaria-containing samples originating from four red deer yielded an amplicon of the expected size in only one case. The 345 bp sequence of this amplicon (GenBank accession nr. KU886068) has 92–93% identity with many *Onchocerca* species, including *O. jakutensis* and *O. flexuosa*. To test whether nuclear pseudo-genes were amplified, the DNA sequences were translated, giving the same level of similarity at the corresponding amino acid sequences of these two species.

4. Discussion

The morphology of the described filarioids is in agreement with the characteristics of the genus *Onchocerca* as given by Bain (1981), i.e. a thickened cuticle in females with transverse annular ridges on

the surface and striae beneath, and weakly developed body muscles. The specimens belong to the species *O. jakutensis* following the description given by Bain (1981) and Demaskiewicz (1993). i.e. oesophagus, shorter than 1000 µm, divided into a muscular and a posterior glandular part, vulva opening slightly posterior to the oesophagus, five pairs of pericloacal papillae, placed before, adjacent to and after the cloaca and two pairs of papillae in the vicinity of the tip of the tail, close to the two phasmids with protruding flaps. Also the size and morphology of the unequal spicules correspond with these descriptions. However, the specimens of *O. jakutensis* examined here differ in some respects from the morphological descriptions given in the literature. It is important to specify these differences, to integrate them into the taxon *O. jakutensis* and to link them with the DNA sequence data from the same material. Partial sequence of the mitochondrial NADH-dehydrogenase gene slightly differed from a corresponding GenBank entry (DQ523771).

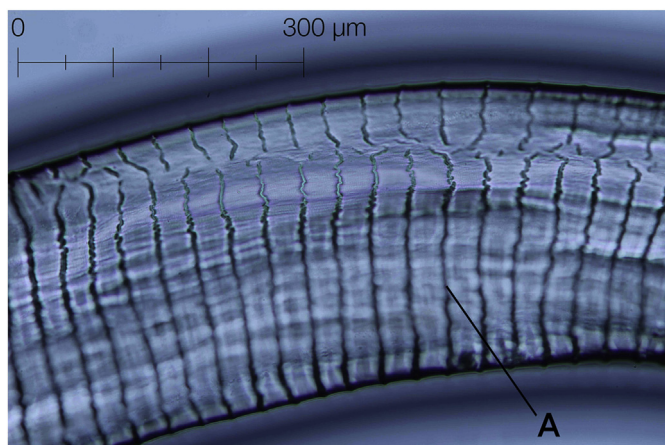


Fig. 5. External cuticular annulation (A) of *O. jakutensis* female with interruption over lateral field.

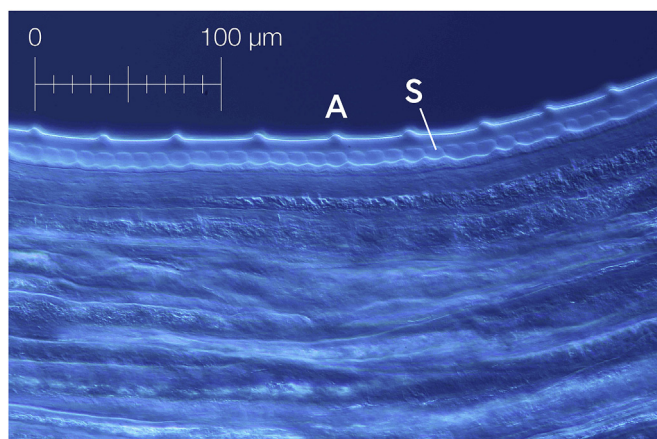


Fig. 6. *O. jakutensis* female: Ratio of cuticular annulation (A) to medullar striae (S) 1:4.



Fig. 7. Microfilaria showing transverse annulation and irregular shape of swollen anterior end (magnitude of annulation and swelling might be exaggerated by artefact of fixation).

Bain (1981) considered the ratio of cuticular annulae to striae in the deeper medulla to be of taxonomic value and used it in the key to the species. She reported a ratio of 1:3 for *O. jakutensis*. Redescribing the species Demaskiewicz (1993) noted “usually” a

ratio of 1:3 but in the corresponding figure the ratio 1:4 was depicted. In their study, Morandi et al. (2011) reported a ratio 1:3 or 1:4. In the present material we observed a ratio 1:4 in all females. In males, both Bain and Schulz-Key (1974) and Bain (1981) reported the presence of an unpaired preloacal papilla, whereas this is not mentioned by Demaskiewicz (1993). In the material examined here, a preloacal papilla is absent (Fig. 4).

With up to 80 cm and more in length, *O. jakutensis* females are long nematodes, their mouth orifice, in contrast, is extremely small. With a diameter of 1.5–9 μm it is smaller than the diameter of its own microfilariae, with the obvious advantage of preventing the offspring from being swallowed by their parents, as was observed to occur in large numbers in another filarioid with a much larger mouth opening, *Oswaldofilaria samfordensis*, living in the serous membranes of agamid lizards (Manzanell, 1982).

Microfilariae found in the eight samples were of different sizes (235–270 μm × 6–10 μm or 380–440 μm × 18 μm), putatively corresponding to *O. jakutensis* and *O. skrjabini* (Schulz-Key, 1975). Genetic analysis of microfilariae in the present study was possible with one of the six samples, namely with the only sample that contained a considerable number of them. A sequence that was distinctly different from those reported from *O. jakutensis* was obtained. No corresponding sequences are available for two of the other *Onchocerca* species that have been described in the same hosts in Central Europe, i.e. *O. skrjabini* and *O. garmsi*. Thus, probably mixed infections occurred, as has been described in a study from neighbouring countries where prevalences in 94 investigated red deer were 96% for *O. flexuosa*, 82% for *O. skrjabini*, and 23% for *O. jakutensis* (Schulz-Key, 1975). Chamois and roe deer in the same area were apparently not infected.

Local hunters have been aware of the presence of subcutaneous nodules in red deer for some time, but misinterpreted them as consequences of tick bites. This is the first record of *O. jakutensis* in Switzerland, and extends its range of distribution from Yakutia in Eastern Russia (Gubanov, 1964, in Demaskiewicz, 1993), Caucasus (Demaskiewicz, 1993), Germany (Bain and Schulz-Key, 1974; Plenge-Bönig et al., 1994), Italy (Morandi et al., 2011) and now also to Switzerland. Despite the relatively small amount of data, the prevalence of *O. jakutensis* is comparable to studies in Germany (Schulz-Key, 1975).

The Swiss red deer population is relatively young. Repopulation started from neighbouring Austria in 1872 (Haller et al., 2002), and this bottle-neck effect of the recolonisation could explain the small distribution range of the parasites and the lower number of species present.

Worldwide, 25 cases of a zoonotic onchocerosis are known. Given that *Onchocerca* spp. are transmitted by blackflies and biting midges, the importance of research on their prevalence and the rates of infection in Switzerland should be emphasised. The impact of this parasite on the deer population and eventually on humans has not been adequately studied.

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